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FACULTY OF NATURAL SCIENCES

BACHELOR THESIS

Cyclophosphamide Effects on Hematopoiesis

Účinky cyklofosfamidu na hematopoesu

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Author's declaration:

I declare that I have written this thesis independently with the help of literature and sources listed. Neither this thesis nor any substantial part of it was submitted with the aim to obtain another or the same academic degree.

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Abstract

Cyclophosphamide is an alkylating agent developed in the 1960s for the use in treatment of cancerous diseases. Since its introduction, it has manifested various spectra of effects. Of uttermost importance are the impacts cyclophosphamide has on the hematopoietic system housed in the bone marrow of femoral and other bones. Hematopoiesis is a complex process which has been extensively studied for decades. The more it is known about the niches the hematopoietic stem cells occupy as well as of their life cycle, the more it is possible to design functional therapy for its malignancies and improve the survival rates. The effects of cyclophosphamide administration on hematopoietic system are divided into three major cathegories: myeloablation and myelosuppression, immunosuppression, and mobilisation of hematopoietic stem cells into the peripheral blood. The immunosuppression is achieved by systematic depletion of progenitors differentiating into lecocytes. Induced neutropenia and lymphopenia allow for management of autoimmune diseases and preservation of transplants. Mobilisation, a process opposite to stem cell homing, seems to be dependent on disruption of cellular adhesion (CRXCR4/SDF-1, VCAM-1/VLA-4) facilitated by proteases released into the environment after cyclophosphamide exposure.

Key words: cyclophosphamide, hematopoietic stem cells, cytochrome P450, cytochrome P450 reductase, immunosuppression, mobilisation, cellular adhesion.

Abstrakt

Cyklofosfamid je alkylační činidlo vyrobené v šedesátých letech minulého století za účelem použití v protirakovinné terapii. Od svého uvedení manifestoval široké spektrum účinků. Velkou pozornost si zaslouží zejména účinky, které cyklofosfamid má na krvetvorbu, jež je lokalizována v dřeni stehenních a dalších kostí. Krvetvorba je složitý proces, jehož výzkum se odehrává už řadu desetiletí. Čím více je toho známo o takzvaných nikách okupovaných hematopoetickými kmenovými buňkami, jakožto i o jejich životním cyklu, tím snadněji se dá vyvíjet účinná terapie proti malignitám, což ve výsledku vede i k navýšení počtu přeživších pacientů. Dopad cyklofosfamidu na krvetvorbu se dá shrnout třemi hlavními pojmy: myeloablace a myelosuprese, imunosuprese, mobilizace hematopoetických kmenových buněk do periferní krve. Imunosuprese je dosaženo systematickou deplecí progenitorových buněk diferencujících se do bílých krvinek. Navozená neutropenie a lymfopenie umožňují zmírnění (i když většinou nikoliv vyléčení) autoimunitních chorob a zabránění odmítnutí štěpu po allotransplantaci. Mobilizace, proces opačný k takzvanému „homingu“ krvetvorných kmenových buněk, se zdá být závislou především na eliminaci buněčné adheze (CRXCR4/SDF-1; VCAM-1/VLA-4) prostřednictvím proteáz vyloučených do prostředí niky po vystavení cyklofosfamidu.

klíčová slova: cyklofosfamid, hematopoetické kmenové buňky, cytochrom P450, cytochrom P450 reduktáza, imunosuprese, mobilizace, buněčná adheze.

LIST OF CONTENT

1. Introduction

N,*N*-bis(2-chloroethyl)-1,3,2-oxazaphosphinan-2-amine 2-oxide (cyclophosphamide) has been used in humane as well as veterinary medicine for great many years (Henry et al, 1994). It is a potent drug belonging into a family of alkylating agents called oxazaphosphorines that have numerous effects. They are: mutagenic, teratogenic, carcinogenic, immunosuppressive and antineoplastic to name just a few (Kenney et al, 2001). Cyclophosphamide is currently in use worldwide mainly in the treatment of several kinds of cancerous diseases, usually in combination with several other chemotherapeutic agents. It is quite versatile as far as its use in the clinical setting is concerned.

Among its most important effects are those cyclophosphamide has on hematopoiesis. Not only is it able to cause myeloablation and immunosuppression, it is also capable of mobilising hematopoietic stem cells into the peripheral blood and thus enables their use for autotransplantation in patients with severe hematologic malignancies and immune malignancies.

This thesis aims to explore and put into perspective the knowledge that has been accumulated and refined since the first preparation of cyclophosphamide some sixty years ago.

2. Cyclophosphamide Metabolism

Cyclophosphamide in itself is a harmless substance (a prodrug) that needs to be bioactivated to acquire its cytotoxic properties (see Figures 4 and 5).

2.1. Key Enzymes

2.1.1. Cytochrome P450

Cytochrome P450 (CYP) is a name given to a group of enzymes belonging to the class of oxidoreductases. The P450 part of the name is derived from the fact that during a spectroscopic analysis they demonstrate the highest absorbance when the analytic beam of light has the wavelength of 450 nanometres.

These enzymes can been found throughout eukaryotic organisms and in some bacteria (generally those, which use hydrocarbons as a source of carbon as well as energy). In mammals they are included in a wide spectrum of metabolic pathways – steroidogenesis, metabolism of vitamins A and D, eicosanoid metabolism, transformation of xenobiotics (de Montellano, 2005). Some of them possess several functions and respond to the presence of a wider range of substrates. Only a handful of human cytochrome P450s are capable of transformation of xenobiotics, but all of those are capable of processing an endogenous substrate, albeit usually with lower specificity and/or metabolic rate.

CYPs are cysteinato-heme proteins (see Figure 1) which in mammals vary in expression according to the species, localisation, environment – some CYPs are expressed constitutively, others are inducible – age and even gender of the individual. The majority of CYPs are in occurrence in the liver. They are membrane-bound proteins which occur predominantly in the endoplasmic reticulum, even though a small enclave can be encountered in the mitochondria.

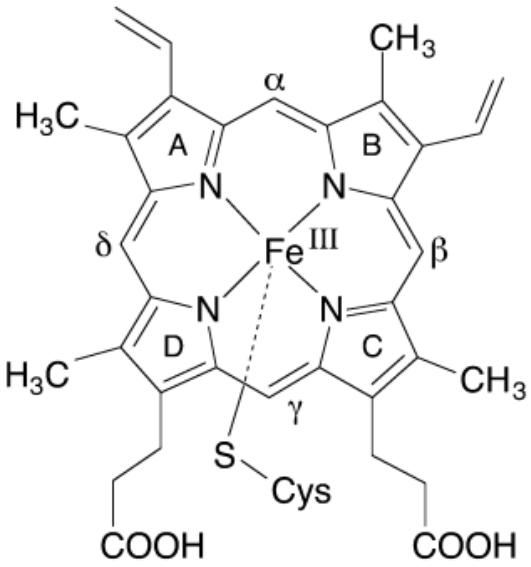

Figure 1. Prosthetic of cysteinato-heme enzymes: an iron-(III) protoporphyrin-IX linked with a proximal cysteine ligand. (reproduced from Meunier et al, 2004)

Cytochrome P450s have been identified as a crucial component of the cyclophosphamide metabolic pathway, as it is these enzymes that enable cyclophosphamide's bioactivation (Pass et al, 2005; Ren et al, 1997). Cyclophosphamide in the liver is activated by a group of CYP isoenzymes by means of hydroxylation on its C-4 atom. This reaction yields 4-hydroxycyclophosphamide and its tautomeric form aldophosphamide, which are at equilibrium.

CYPs are often referred to as monooxygenases because they use molecular oxygen in the oxidation reactions they catalyse, and incorporate only one of its atoms into the substrate that is being catalysed. The other oxygen atom is reduced to water with two electrons provided by NAD(P)H via a reductase (Equation 1).

$$\mathbf{RH + O_2 + 2H^+ + 2e^- \longrightarrow ROH + H_2O}$$

Equation 1. Monooxygenation catalysed by a cytochrome P450 enzyme.

The enzymes thought to possess the greatest potential with regards to cyclophosphamide metabolism have been identified as: CYP2B6, CYP2A6, CYP2C9 and CYP3A4 (Roy et al, 1999). While the majority of the applied dose of cyclophosphamide follows the route of 4-hydroxylation and activation, a smaller portion can follow another metabolic pathway – N-dechloroethylation and subsequent formation of inactive and neurotoxic chloroacetaldehyde. CYP3A4 proved to be the most prolific in this alternative metabolism (Huang et al, 2000).

Unfortunately, there is a substantial genetic polymorphism in CYPs, leading to several abnormalities in the metabolism of cyclophosphamide – such as lower substrate specificity, a lower metabolic rate – in some individuals, which may result in the patient being less responsive to treatment by the drug, and other adverse situations (Griskevicius et al, 2003; Xie et al, 2003).

Moreover, cyclophosphamide is often used in combination chemotherapy during which several chemicals are co-administered, therefore possible drug-drug interactions have to be carefully evaluated. For example triethylenethiophosphoramide (thioTEPA) – a common antineoplastic agent – is responsible for specific uncompetitive inhibition of CYP 2B6, and thus interferes with the conversion of cyclophosphamide to 4-hydroxycyclophosphamide (Harleton et al, 2004; Rae et al, 2002). Even cyclophosphamide itself regulates its own metabolism by induction of CYPs with subsequent increase in 4-hydroxylation rate (Chang et al, 1997); on the other hand acrolein that is produced further along the activation pathway is thought to be responsible for inhibiting cyclophosphamide metabolism by interacting with both CYPs and the CYP reductase (LeBlanc & Waxman, 1990).

2.1.2. CYP Reductase

Cytochrome P450 reductase (also known as cytochrome c reductase) is a protein localised on the cytosolic face of the endoplasmic reticulum, and is most abundant in hepatocytes (Phillips & Langdon, 1962). It is safely anchored into the membrane of the endoplasmic reticulum by a sequence of about sixty amino acids. The cytosolic part is comprised of two domains. The first one has two binding sites for NADPH and uses a FAD molecule as a cofactor, while the other one employs a

FMN (flavin mononucleotide) molecule as its cofactor and is responsible for the contact with a CYP molecule. The tertiary structure of the enzyme is highly conserved, indicating its importance.

The reductase shuttles electrons to the CYP in several steps. Upon binding NADPH the FAD-domain is reduced by two hydride ions simultaneously. In the next step, the electrons are channelled to the FMN-domain in sequence, and finally to the CYP (Vermilion et al, 1981). Two conformational states of the reductase have been described – the "open" state is compatible with FMN-heme electron transfer, while the "closed" state allows FAD-FMN electron transfer (Fig. 2) (Ellis et al, 2009).

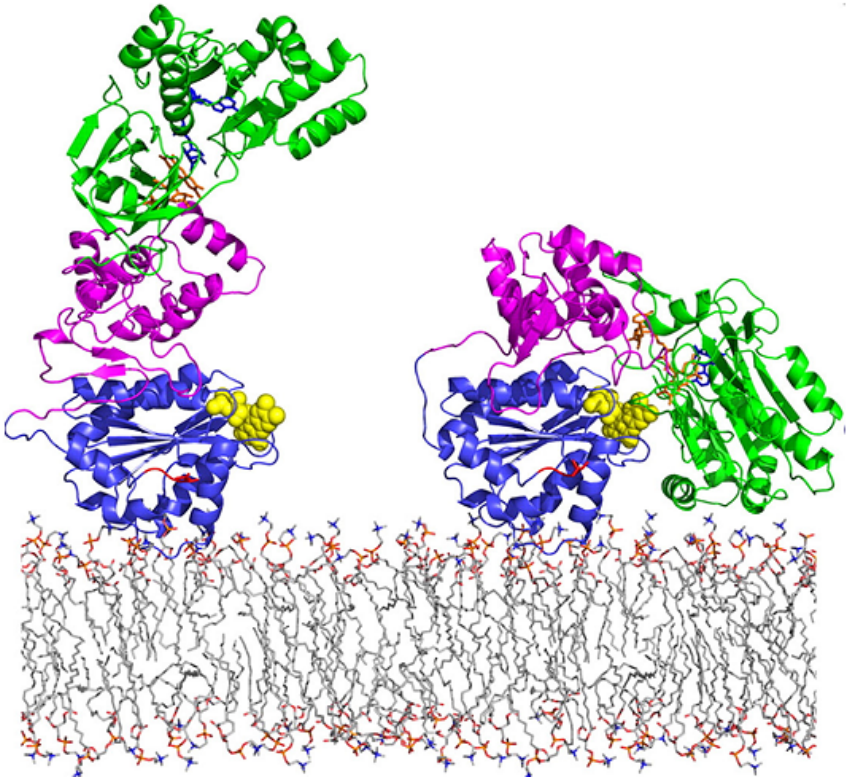

Figure 2. The open (left) and closed (right) conformations of the CYP reductase. The FMN domain is shown in blue, the linker domain in purple and the FAD domain in green colour. The cofactors are shown: FMN – yellow, FAD – red, NADPH – blue (reproduced from Ellis et al, 2009).

Despite having two binding sites for NADPH, only one of them is catalytic. Simultaneous binding of two molecules of NADPH actually attenuates the reaction,

most probably by interfering with the release of $NADP^+$ from the catalytic site (de Montellano, 2005).

The diflavin reductase is vital for monooxygenation reactions of CYPs because it provides the electrons necessary for reduction of the CYP in question, and subsequently the reduction of an oxygen atom to water. An experimental knockout mouse strain deficient in expression of hepatic CYP reductase (Hepatic Cytochrome P450 Null – HNR) – to the extent of expressing less than 1 % of the amount of the enzyme produced by wild-type mice – in which has the conversion of cyclophosphamide to 4-hydroxyphosphamide been significantly halted, and its half-life in plasma dramatically increased, have been used to study the importance of the CYP reductase for CYP-facilitated drug metabolism (Pass et al, 2005). Despite having virtually no hepatic CYP reductase activity, the bioactivation of cyclophosphamide still takes place owing to extra-hepatic activation, but the amount of 4-hydroxycyclophosphamide produced is correspondingly lowered. The extra-hepatic bioactivation of cyclophosphamide has been demonstrated on another mouse strain – the CL-LCN (CYP reductase-low, liver- CYP reductase-null) mice (Gu et al, 2007).

2.2. Activation Pathway

2.2.1. 4-hydroxycyclophosphamide

A tautomer of aldophosphamide, 4-hydroxyphosphamide has been shown to possess little cytotoxic potential and is therefore not considered to be a therapeutic metabolite of cyclophosphamide. Nevertheless, it does have an important function as this is the form in which the drug enters target cells, and tautomerises to aldophosphamide, which then undergoes further metabolic processing leading to the final therapeutic product. It is presumed that 4-hydroxycyclophosphamide travels to its final destination bound to the red blood cells. This notion is largely supported by the findings of higher concentrations of activated oxazaphosphorines in the erythrocytes than in the plasma (Highley et al, 1997).

2.2.2. Spontaneous Reaction of Aldophosphamide

Aldophosphamide that is transported to target tissues by the blood circulatory system undergoes a non-enzymatic elimination to form phosphoramide mustard and acrolein, former of which is an alkylating agent that forms intra- as well as interstrand cross-links in the DNA and protein-DNA cross-links. Such an intervention to the integrity of the DNA constitutes a significant damage to the cell and may lead to its death by either apoptosis or necrosis – that is the basic principle of cyclophosphamide and related substances' (ifosfamide, trofosfamide) therapeutic effect. On the other hand, acrolein is a highly nephrotoxic and neurotoxic by-product of the reaction. Cytotoxic properties of the compound cause a disease of the urinary bladder called haemorrhagic cystitis (Lawrence et al, 1975).

Even though the reaction does not require a catalyst, it has been established that human serum albumin speeds up the process (Kwon et al, 1987). However, the phosphoramide mustard generated in the blood does not pose a significant threat to tissues as it is mostly ionised (at physiological pH) which is the underlying reason for its decreased ability to pass through the plasma membrane of cells.

In addition to the spontaneous reaction aldophosphamide can also serve as a substrate for an aldehyde dehydrogenase which catalyses its oxidation to the inactive carboxyphosphamide by molecular oxygen. At the same time a superoxide anion belonging to the group of reactive oxygen species (ROS) is generated. It has been suggested that upregulation of the enzyme contributes to cyclophosphamide resistance (Manthey et al, 1990).

2.2.3. Acrolein

In spite of contributing to the cytotoxic properties of cyclophosphamide, the generation of acrolein quantitatively equal to phosphoramide mustard is mostly undesirable due to its role in the development of haemorrhagic cystitis and neurotoxicity. Acrolein produced by β-elimination from aldophosphamide causes single-strand breaks in the DNA and readily binds to cellular proteins (Crook et al, 1986). From the fact that it has been known to deplete the cellular glutathione has been derived the practice of co-administration of protective drugs which include

sulphhydryl-containing compounds such as mesna (Figure 3), which allow for its safe elimination from the body.

Acrolein is at least on order of magnitude more effective in depletion of glutathione than phosphoramide mustard, therefore agents responsible for protection again acrolein toxicity can be used without halting the curative effect of cyclophosphamide (Gurtoo et al, 1981).

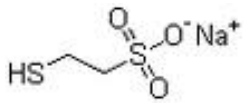

Figure 3. The structure of mesna.

2.2.4. Phosphoramide Mustard

The phosphoramide mustard's potential to cause damage to cells by alkylation of their nucleic acids lies within its electrophilic aziridinium group which naturally seeks a nucleophilic partner to react with. The N-7 of guanines has proven to be a favoured site for alkylation, especially if they are flanked by more guanines. As the mustard possesses two functional groups, it interconnects two reaction sites, thus forming the inter- and intrastrand (most often between directly adjacent bases) crosslinks as well as nucleic acid-protein crosslinks (Kohn et al, 1987).

Cyclophosphamide metabolism has been extensively studied in mice. There is a major difference between human and murine metabolisms that has to be accounted for when applying murine data into clinical practice. While there are no active metabolites detectable in murine blood as soon as one hour after dose administration (personal communication with RNDr. Luděk Šefc, CSc.), the build-up of active metabolites and clearance in humans is slower and happens in a space of several hours. Importantly, there does not seem to be a difference between oral and intravenous routes of administration, which means patients can be offered the more comfortable oral treatment without compromising the therapeutic outcome (Struck et al, 1987).

Cyclophosphamide and its metabolites are mostly cleared from the body via urine.

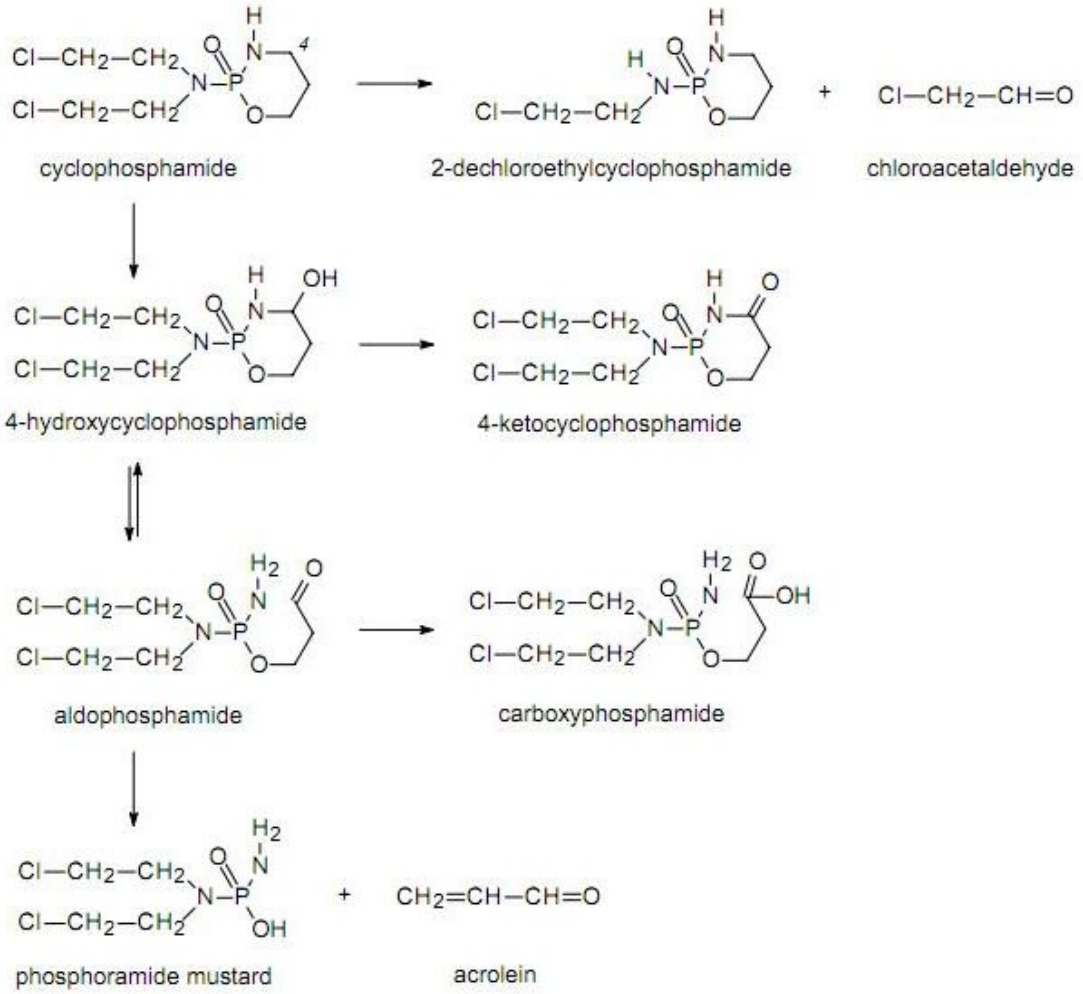

Figure 4. Metabolism of cyclophosphamide. Vertically: activation of cyclophosphamide; horizontally: inactivation pathways (reproduced from de Jonge et al, 2005).

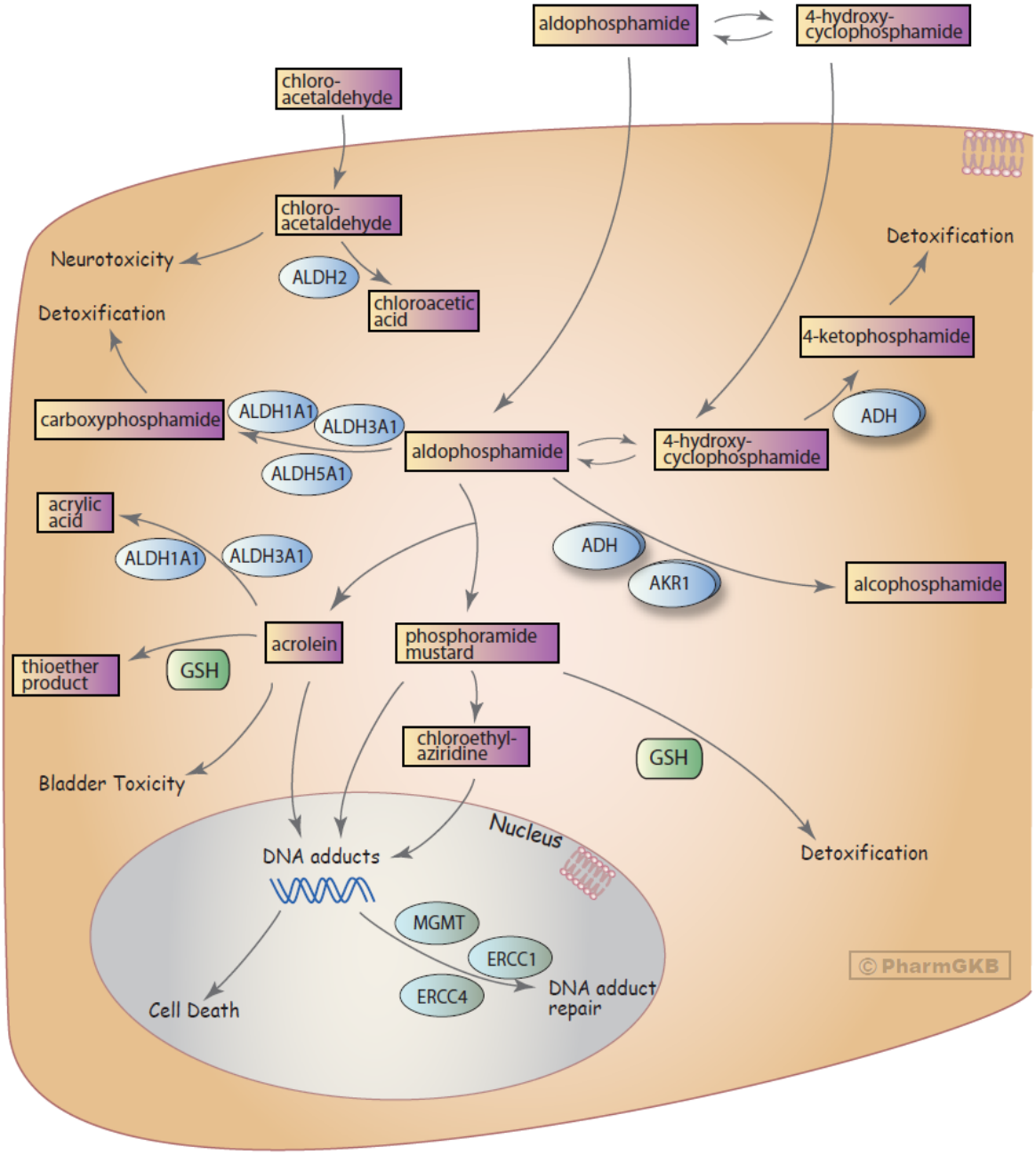

Figure 5. Metabolic pathways of cyclophosphamide in a target cell. (source: http://www.pharmgkb.org/views/pathway/PA2034.pdf - reproduced with permission from PharmGKB and Stanford University)

3. Hematopoiesis

Hematopoiesis is a process during which hematopoietic stem cells (HSCs) maintain their numbers, and produce a cohort of terminally differentiated mature blood cells. During the foetal development hematopoietic activity can be observed in the liver as well as the spleen, but the bone marrow is the only site that supports hematopoiesis after the birth and throughout adulthood. Indeed, extramedullary (occurring outside the bone marrow compartment) hematopoiesis is widely recognised as a sign of a disease and/or a direct consequence of chemotherapeutically or by irradiation impaired hematopoietic capacity of the bone marrow. In these cases, extramedullary hematopoiesis serves as a mechanism for compensation of the lost medullary hematopoiesis (Singer et al, 2004). Splenectomised mice recover more slowly than controls after cyclophosphamide-induced hematosuppression (Wang et al, 2009).

HSCs differ from the embryonic stem cells in one important aspect – they cannot give rise to the whole spectrum of cell types occurring in the body. The HSC is a member of a family of tissue-specific stem cell, among which are also counted neural stem cells, cord blood stem cell from the umbilical cord, bone marrow stromal stem cells. As the name "tissue-specific stem cells" suggests, these stem cells are only used to replenish specific types of tissues. They are multipotent.

Special places within the bone marrow housing HSCs have been postulated. These are called niches, and currently two distinct types of them are recognised. The niche is a place in which a HSC receives signals that regulate its function or induce dormancy, be it by soluble metabolites, cytokines or direct cell-to-cell contact with non-HSCs forming the niche. Each heamatopoietic stem cell can upon division follow either of two routes – (a) maturation through sequential loss of potency and exit from the niche (b)staying in place and continuing with the production of its own clones or entering the state of quiescence.

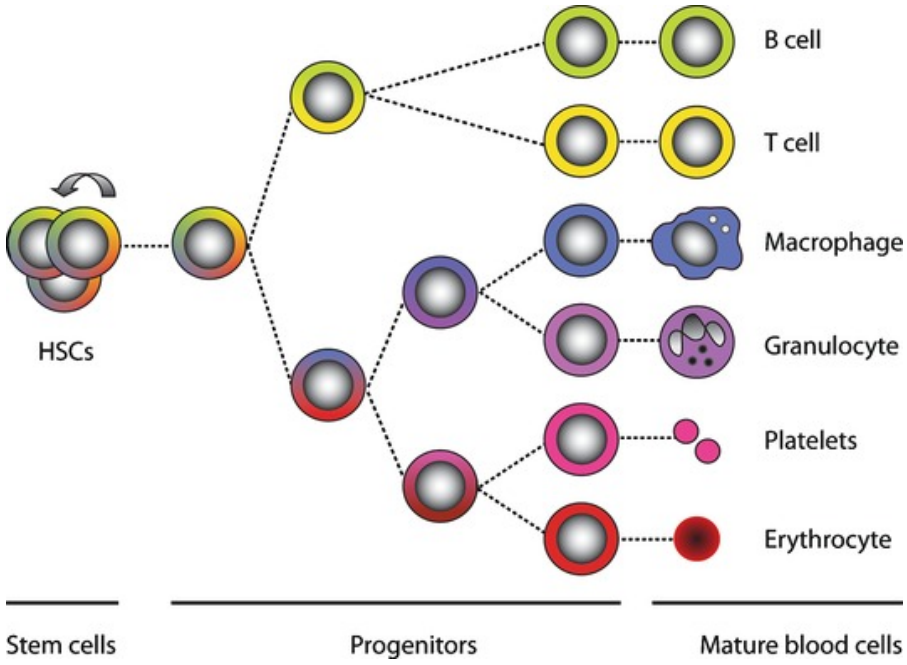

Figure 6. A simplified overview of hematopoiesis. The self-renewal capacity of HSCs is indicated by the arrow. The gradual loss of colouring indicates increased lineage commitment (Gerrits et al, 2008).

Of the two aforementioned types of niches, the endosteal niche seems to be the place where dormant and slow cycling HSCs with the biggest potential for self-renewal are located. The niche comprises cells of mesenchymal origin which directly interact with the HSCs. The HSCs residing in this niche are called long-term hematopoietic stem cells (LT-HSCs), which reflects their ability contribute to hematopoiesis over a long period of time or even a lifetime.

The microenvironment which is typical for this niche is made up by stromal cells – osteoblasts, adipocytes, endothelial cells and fibroblasts, and the endothelium is in direct contact with the calcified bone matter. A population of specialised N-cadherin-expressing osteoblasts (SNOs) has been found to be absolutely essential for HSCs. These SNOs communicate with the HSCs through homotypic N-cadherin interactions (Zhang et al, 2003). Experiments with mice that had the numbers of bone marrow osteoblasts dramatically lowered (conditional or permanent ablation) showed that these mice had also insufficient numbers of LT-HSCs and, as a direct consequence of that, substantial decrease of lymphoid, erythroid and myeloid

progenitors (including osteoclast progenitors) was observed (Visnjic et al, 2004). The interactions in the niche do not seem to be only one-way, as it has been noted that HSCs drive mesenchymal cells toward osteoblastic lineage when they are grown in a co-culture.

The other distinct type of niche is a vascular one. This niche is supposedly inhabited by short-term hematopoietic stem cells (ST-HSCs) that have migrated here from the endosteal niche. As is to be expected, the architecture and overall microenvironment of the niche differs from the endosteal one. In the vascular niche the HSCs are in contact with the fenestrated endothelium of capillaries, which enables them to closely monitor concentrations of molecules dissolved in blood. Therefore a swift response could be mounted should the situation require it (Yang et al, 2005).

The ST-HSCs give rise to a progeny of multipotent progenitor cells (MPPs). The MPPs can support generation of mature blood cells of all types, but lack the ability to self-renew. The ST-HSCs have in contrast to the LT-HSCs a significantly lower capacity for self-renewal and consequently can single-handedly support hematopoiesis for several weeks only, which is why LT-HSCs are by far better suited for hematopoietic stem cell transplantations. HSCs transplantations are typically carried out in an attempt to correct aberrant hematopoiesis, therefore a long-term solution to the patient's problems is desirable.

Hematopoiesis is complex process, regulation of which is not yet fully clarified, even though a great mass of processes has been studied and our knowledge of it is quite extensive. Apart from direct cell contact and communication between endosteal and vascular niches, cytokines, the endocrine system (regulation of erythropoiesis by erythropoietin) and metabolic factors (such as varied oxygen levels) all take part in it. For example granulocyte colony-stimulating factor (G-CSF), granulocyte-monocyte colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF) are all powerful stimulators driving hematopoietic progenitors to differentiation *in vitro*. However, *in vivo* deficiencies in these factors do not necessarily lead to a certain cell type being completely absent (Lieschke et al, 1994). It seems that these factors do not solely provide lineage-specific signals for

differentiation of HSCs and multipotent progenitors, but rather stimulate the survival/expansion of progenitors, and play a greater role at the terminal maturation stages. On the other hand, interleukin-7 (IL-7) is utterly irreplaceable when it comes to B-cell differentiation. Mice deficient in IL-7 still do produce common lymphoid progenitor cells (CLPs). B-cell production, however, is blocked. The IL-7 regulates the immunoglobulin gene rearrangement once the cell has committed to the B-cell lineage and entered the pro-B-cell stage (von Freeden-Jeffry et al, 1995).

Hematopoietic stem cells are seldom to be seen circulating in the blood (Abkowitz et al, 2003). During the period of embryonic development they migrate from liver and "home" into the bone marrow, establish a stable population in there, and produce high levels of immature and maturing cells of all lineages. The HSCs can be encountered in blood during leukemias and various inflammatory conditions, after injection of granulocyte colony-stimulating factor (G-CSF), and after administration of cyclophosphamide (Szumilas et al, 2005).

3.1. Cyclophosphamide Effects on Hematopoiesis

3.1.1. Myeloablation, Myelosuppression and Immunosuppresion

Cyclophosphamide was developed in the 1960s with the objective to create a powerful new antineoplastic agent that would be maximally efficient in battling cancerous growth and, at the same time, be as little toxic to the patients' healthy tissues as possible. Although it has turned out to be pretty competent in the first field, problems connected to its overall toxicity do occur. As it is the case for virtually any antineoplastic drug and radiation therapy, the regimen affects fast-dividing cells the most – probably due to the DNA being more easily accessible when the nuclear envelope dissolves during mitosis – unfortunately neoplastic tissues are not the only ones which divide fairly quickly. The hematopoietic system housed in the bone marrow is another great target for anti-cancer therapeutics, with hematopoietic progenitor cells being the most vulnerable (DeWys et al, 1970; Valeriote et al, 1968). Severe Myelosuppression is to this day one of the key dose-limiting aspects of cyclophosphamide administration in the clinical setting, and might even happen to be a reason for discontinuing chemotherapy in some patients (Presant & Klahr, 1977).

Injury to the bone marrow by a cyclophosphamide-containing regimen of treatment usually leads to a pronounced decline in the granulocyte, lymphocyte, thrombocyte numbers (Fried & Johnson, 1968; Mosienko et al, 2002), and severe cases of anemia, which is in accordance with an observable decrease in bone marrow cellularity (Sefc et al, 2003). While the cases of developed anemia and thrombocytopenia can be confronted by administering blood and thrombocyte transfusions respectively, the transfusions are still just a way of minimising the damage without solving the underlying problem.

The most serious of all cytopenias induced by cyclophosphamide is neutropenia. Patients who see their neutrophil levels fall below the value of 100 cells/mm^3 (severe neutropenia) are extremely prone to infection by pathogens such as *Klebsiella pneumonie*, and are incapable of initiating an efficient immune response against them. A concomitant administration of prophylactic antibiotic drugs is therefore required (Tjan-Heijnen et al, 2001). Treatment protocols also sometimes include G-CSF as it has been shown that it can reduce neutropenia (Neidhart et al, 1989).

In a stark contrast to the undesired chemotherapy-induced immunosuppression in cancer sufferers is the intentional prescription of cyclophosphamide which aims to achieve this result. Cyclophosphamide is a drug of choice for the management of some very severe immune disorders (Burt et al, 1998). Among these are systemic lupus erythematosus (Katsifis et al, 2002), systemic sclerosis; and cyclophosphamide is currently being tested as a promising therapy for autoimmune aplastic anemia patients (Brodsky et al, 2004; Brodsky et al, 2001).

Yet another situation in which immunosuppression by cyclophosphamide is desirable is the pre-transplantation conditioning of the intended host (Kapoor et al, 1981; Litzow et al, 2002). Elimination of host alloreactive lymphocytes serves as a prevention of the rejection of a transplant (Aversa et al, 1999). Moreover, high-dose cyclophosphamide seems to be quite efficient even in the capacity of single-agent prophylaxis against both acute and chronic graft-versus-host disease (Luznik et al).

The myelosuppressive effect has been attributed to cyclophosphamide right from the very beginning of its use in cancer therapy. Mice that were subjected to cyclophosphamide in doses equivalent of bone marrow-injury causing γ-irradiation in early studies had been found to develop a corresponding response of a trauma and subsequent improvement. What was more of an interest was the fact that murine bone marrow and peripheral blood cell count recovered more swiftly after cyclophosphamide regimen. Moreover an overshot in reticulocytes could be observed – so-called rebound reticulocytosis (Valeriote et al, 1968). While the myelotoxic effect of cyclophosphamide has an unquestionably great negative impact on experimental models (mostly mice) and human patients both, it is nonetheless only temporary and can be weathered with supportive therapy.

The transient nature of cyclophosphamide-induced myelosuppression is attributable to its mode of action. While rapidly proliferating hematopoietic progenitor cells are hit with full force, slow-cycling hematopoietic stem cells are more resistant to the drug. The conservation of HSCs is intrinsic for hematopoietic reconstitution. Cyclophosphamide regimens need to be established so that HSCs have enough time to replenish the pool of HPCs. During the period of hematopoietic reconstitution, when the HSCs divide rapidly to compensate the loss of progenitor and mature blood cells, are even these cells susceptible to damage by cyclophosphamide, which is counterproductive in regimens where the HSCs are not the intended targets.

In recent years, various studies have been focused on eliminating, or at least decreasing, the hematologic toxicity of chemotherapeutic agents. Notably macrophage inflammatory protein-1 alpha (MIP-1α) piqued researchers' interest due to its ability to cause cell-cycle arrest, which could serve as protection for HPCs. However, this chemokine naturally polymerises in extremely low concentrations (>20 ng/ml), which consequently renders it inactive. Retaining its native form by means of organic solvents was not a suitable option for patient therapy. A recombinant protein with significantly lowered polymerising tendencies has been developed. While it cannot prevent cyclophosphamide-induced neutropenia or its

severity, it seems to speed up the recovery process, which is promising for the future (Gilmore et al, 1999; Marshall et al, 1997).

Meanwhile cyclophosphamide serves as an effective means of making murine models immunosuppressed, which is very useful for exact evaluation of the efficacy of antibiotics (Zuluaga et al, 2006).

3.1.2. Mobilisation of Hematopoietic Stem Cells

The list of effects cyclophosphamide has on hematopoiesis by no means ends at myelosuppression and immunosuppression. Another important outcome of cyclophosphamide administration is the mobilisation of hematopoietic stem and progenitor cells (sorted according to the expression of surface $CD34^+$ marker) into the peripheral blood. This is widely used in clinical practice for mobilisation of $CD34^+$ cells into the peripheral blood, from where they can be collected by a process of apheresis and further used for autologous transplantations (Rosen et al, 2000). Mobilisation protocols do not usually employ cyclophosphamide as the sole agent as it has been demonstrated that significantly higher numbers of progenitors are collected when G-CSF or GM-CSF are included in the regimen (Caporali et al, 2001). The aforementioned growth factors can also stimulate mobilisation singlehandedly (Abkowitz et al, 2003), but their combination with chemotherapy is also beneficial in terms of yield. Nevertheless, cyclophosphamide is not included in mobilisation regimens in healthy donors due to its toxic properties, because it would simply not be ethical to impose those on healthy individuals.

The process of release of HSCs and HPCs from the bone marrow into the peripheral blood has not yet been completely elucidated, although some important discoveries have been made. Being de facto the direct opposite of homing, the attention of researches has been turned to adhesive molecular pairs such as very late antigen 4 (VLA-4) and vascular cell adhesion molecule-1 [VCAM-1 (Kikuta et al, 2000)]; and chemokines and their receptors such as stromal cell-derived factor 1 (SDF-1/CXCL12) and its receptor CXCR4. The evidence for the importance of VLA-4/VCAM-1 stems from studies utilising anti-VLA-4 antibodies. The administration of VLA-4 antibodies leads to successful mobilisation of HSCs in

experimental animals (Papayannopoulou & Nakamoto, 1993). Neutralisation of VLA-4 also prevents homing of transplanted HSCs (Zanjani et al, 1999).

SDF-1 is highly expressed in the bone marrow, notably by the immature osteoblasts of the endosteal niche. The gradient of SDF-1 is deemed necessary for homing of $CD34^+$ both in steady-state hematopoiesis and after transplantation into myeloablated hosts. CXCR4 is a membrane-bound receptor protein with seven transmembrane domains. It is linked to G-proteins and the signal cascade initiated upon interaction with SDF-1 may result for example in the reorganisation of actin cytoskeleton and formation of pseudopodia. SDF-1α induces phosphorylation of multiple adhesion proteins. The importance of SDF-1/CXCR4 axis for hematopoiesis has been demonstrated *in vivo* when knock-out mice lacking both molecules developed severe defects in B lymphopoiesis and myelopoiesis.

On the other hand, mobilisation can be induced in mice by artificial overexpression of SDF-1, as too high a concentration leads to desensitisation and internalisation/degradation of CXCR4 of the target cells and thus to their unresponsiveness to SDF-1 (Alsayed et al, 2007). This discovery was most important as it had been previously shown that chemotherapy tends to increase the levels of SDF-1 in the bone marrow, and yet cyclophosphamide does facilitate mobilisation, which naturally seemed contradictory. Moreover, so-called "poor mobilisers" in whom achievement of target numbers of collected $CD34^+$ cells for autologous transplantations is problematic do express more SDF-1 at steady state than "good mobilisers" and healthy individuals (Gazitt & Liu, 2001). Even though mobilised HCSs express significantly less CXCR4 (Carion et al, 2003; Gazitt & Liu, 2001), they are able to home into the hosts bone marrow. This is attributed to chemokine and overall environment of the hosts circulatory system which promotes the production of new CXCR4 receptors on the cell surface.

It has been observed that rising numbers of hematopoietic progenitors in the peripheral blood are in direct correlation with increased levels of proteolytic enzymes in the bone marrow (Levesque et al, 2002). Matrix metalloproteinase 9 (Carion et al, 2003), cathepsin G and leucocyte elastase all degrade the N-terminus of

SDF-1 *in vitro* which disallows binding to the receptor and results in the loss of its chemoattractant function.

AMD-3100 (also known as plerixafor) is a substance causing reversible inhibition of CXCR4 (Hendrix et al, 2000). When it is combined with G-CSF in human donors for mobilisation, the collected cells express higher levels of VLA-4, tend to migrate to SDF-1 better, and also show significantly better engraftment in NOD/SCID (non-obese diabetic/severe combined immunodeficiency) hosts. This example of crosstalk between chemokines and/or adhesion molecules is not the only of the kind, and much effort is currently being put into deciphering the relationship status of various chemokine/adhesion molecule interaction-induced responses of HSCs (Peled et al, 2000).

4. Conclusion

Cyclophosphamide is an old drug put to new uses as we gradually discover how a human body works. Its precise metabolism and mode of action has long been a mystery, but decades of research are slowly heading to illumination of the principles.

It is now possible to fully appreciate how a polymorphism in a gene coding a particular enzyme influences the metabolism of the drug, and interpatient differences can be addressed. As the mankind sees the numbers of malignancies rising, it is most beneficial that cyclophosphamide's mode of action is better understood than it used to be fifty years ago.

Its use in hematology has been extensively studied. Interestingly, cyclophosphamide can mimic both the impact of γ-irradiation and of the hematopoietic growth factor G-CSF depending on dosage, timing and genetic background of the subject.

In its capacity of mobilisation-inducing drug cyclophosphamide demonstrated quite similar tendencies to G-CSF – after its administration, bone marrow environment reacts by releasing stored neutrophil proteases which cleave molecules facilitating chemotaxis and adhesion to the bone marrow niche, thus enabling the egress of stem and progenitor cells into the peripheral blood. Among the affected molecular pairs studied in recent years are CXCR4 and its ligand SDF-1, and VLA-4 with VCAM-1. These interactions cannot be viewed as isolated systems, as one stimulus can often influence the way another one is processed, and there is significant amount of cross-talk going on. More studies of these kinds of interactions are yet to come and will hopefully lead to improvement of clinical protocols and brighter outlook for patients with hematologic and immune malignancies, whose numbers are rapidly growing.

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